Tyrosine kinase assays adapted to homogeneous time-resolved fluorescence

Alfred J. Kolb, Paul V. Kaplita, David J. Hayes, Young-Whan Park, Christine Pernell, John S. Major and Gérard Mathis

Tyrosine kinases are important therapeutic targets in drug discovery high-throughput screening programs. Homogeneous time-resolved fluorescence (HTRF) eliminates many of the problems associated with some conventional screening assay methodologies and this has made the development of tyrosine kinase HTRF assays an exciting prospect for pharmaceutical companies. The authors illustrate the utility of this technology in the light of experience within five pharmaceutical companies.

The family of tyrosine kinase enzymes has been of particular interest as a therapeutic target because of the regulatory control exhibited over a wide range of biological functions, including mitogenesis and transformation. As this target is so important and as there is such a variety of kinases, estimated at ~1,000 different kinases in mammalian cells[1], many pharmaceutical companies consider this a long-term target that will be assayed against many test compounds. Currently used assay methods for kinases, while successfully implemented, have drawbacks that hinder a screening laboratory in meeting the required throughput goals. This has made the tyrosine kinase family a good candidate for conversion to homogeneous time-resolved fluorescence (HTRF®, Packard Instrument Company).

Box 1. Contributing research teams

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The most popular methods for screening tyrosine kinase inhibitors are in-plate binding assays and radiometric assays. The in-plate binding assays include enzyme-catalyzed colorimetric[2] and luminescent[3] read-outs and time-resolved fluorescence[4]. These methods require plate coating and multiple wash and incubation steps, which limits throughput. The radiometric assays using ^{33}P or ^{32}P, although extremely sensitive, can require filtration[5], which

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is labor intensive and relatively slow. A higher throughput radioisotopic method is SPA (scintillation proximity assay, Amersham International)[6] as it does not require separation steps. However, because the amount of radioactivity used in the assay is kept to a minimum for cost and safety reasons, the measurement times can be the rate-limiting step in throughput. HTRF eliminates many of the difficulties associated with colorimetric and radioisotopic methods. As HTRF is homogeneous and occurs completely in solution, there is no coating of plates or solid supports and no separation steps are required. The time-resolved measurement eliminates background fluorescence and the reagents do not require special handling, monitoring or disposal. Moreover, each microplate well is measured in one second to further increase throughput.

The data for tyrosine kinase HTRF assays presented in this review are the result of assay development and high-throughput screening (HTS) that has been initiated at five pharmaceutical companies (Boehringer Ingelheim Pharmaceuticals, Glaxo Wellcome R&D, Merck Research Laboratories, Rhône-Poulenc Rorer and Zeneca Pharmaceuticals) in collaboration with Packard Instrument Company and CIS bio international (Box 1). The results show that HTRF has the biological characteristics, sensitivity and throughput required for the demanding environment of drug discovery. It also compares favorably with non-separation radiometric assays and heterogeneous (in-plate binding) time-resolved fluorescence.

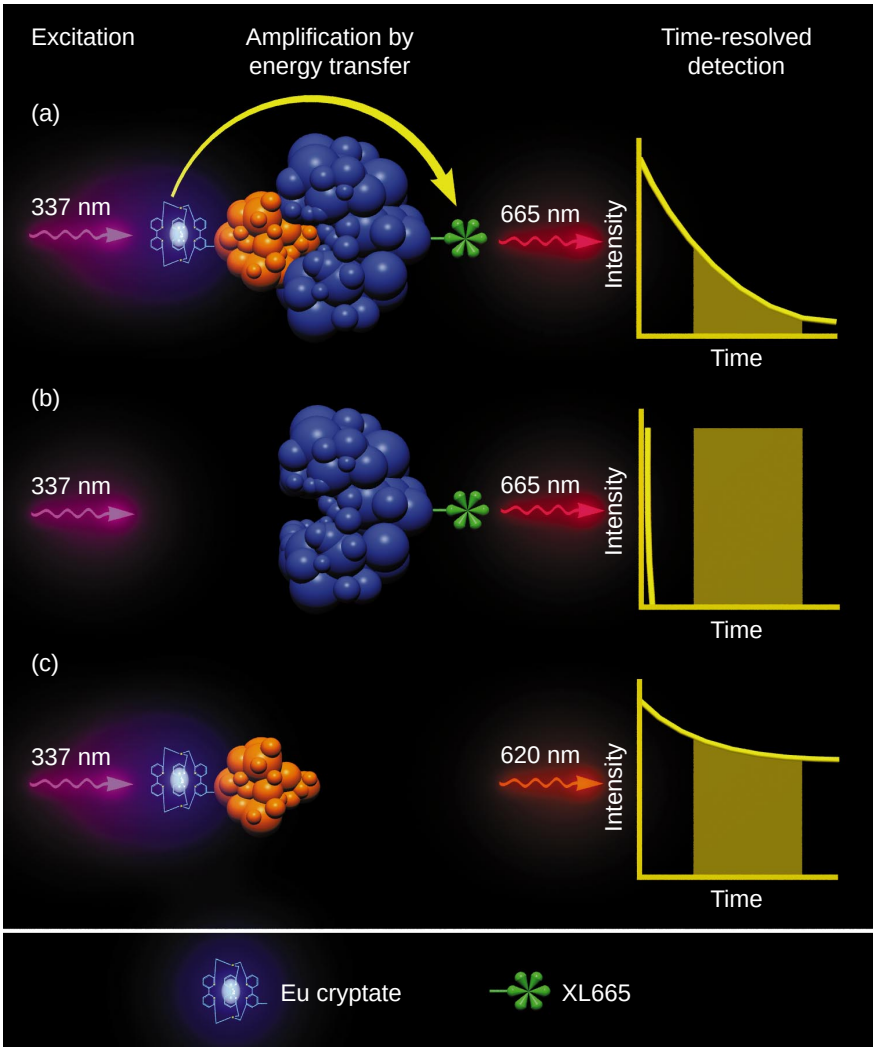

Figure 1. *Principles of HTRF measurements. (a) When europium cryptate (EuK) and XL665 are proximal, a slow decay signal at 665 nm is measured. (b) Distal (unbound or free) XL665 has a prompt decay, like other background fluorescence, and is eliminated by the time-delayed measurement. (c) Unbound EuK is measured at 620 nm and is used as an internal reference for the calculation of the 665 nm/620 nm ratio.*

Principles of HTRF measurements

HTRF is a homogeneous assay method that uses fluorescence resonance energy transfer between two fluorophores. Europium cryptate (EuK) is the donor fluorophore and XL665 (a modified allophycocyanin) is the acceptor fluorophore. Figure 1 illustrates the principles of HTRF (Refs 7–10); part 1a shows the complete HTRF process when two biomolecules labeled with the fluorophores are in proximity (or bound). The EuK absorbs the energy of the laser at 337 nm. It then transfers its energy by non-radiative, direct energy transfer (no light is emitted) to the XL665, which emits at 665 nm with a slow decay time. Prompt decay background fluorescence is eliminated because the XL665 is measured after a 50 µs time delay.

As HTRF requires no separation steps, free (unbound) XL665-labeled biomolecules and free EuK-labeled biomolecules are also present during the measurement. Figure 1b shows that free XL665, which also decays at 665 nm, can be disregarded because it has a prompt decay. Free EuK (Figure 1c) is an important part of the HTRF measurement. It exhibits a slow decay at 620 nm and is measured after a time delay. The EuK signal is used as an internal reference to calculate the ratio of the emission signals. This ratio (counts at 665 nm/counts at 620 nm) is used to compensate for color absorption, turbidity and variations in volume and excitation energy[7,8]. The energy transfer process between EuK and XL665 occurs with 50% efficiency at a distance of 9 nm. The efficiency is inversely proportional to the distance ($1/d^6$). These distances are sufficient to measure biomolecular interactions.

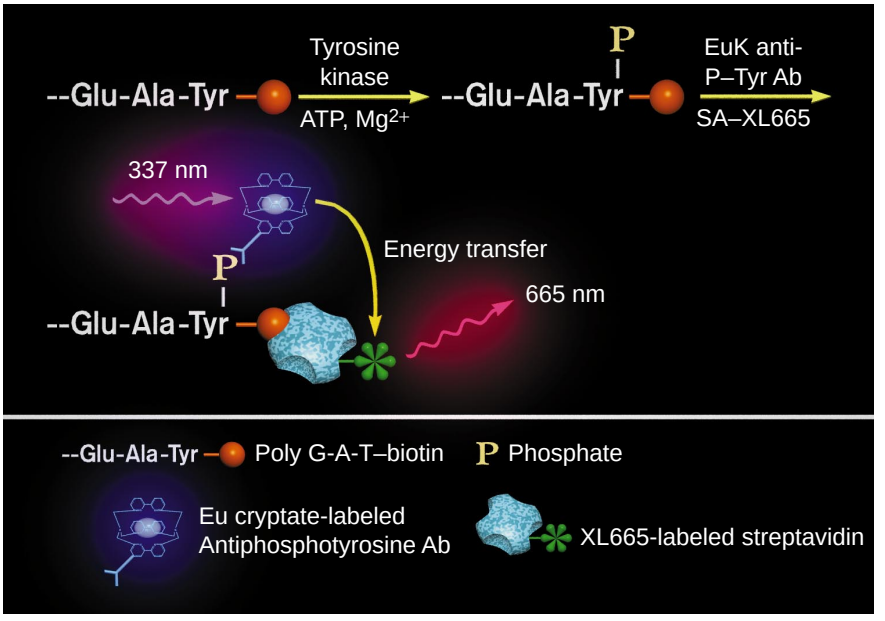

Figure 2. *A typical assay labeling strategy for a tyrosine kinase assay. A biotinylated poly-Glu-Ala-Tyr is one of several substrates that have been used. After the kinase reaction, a europium cryptate-labeled antiphosphotyrosine antibody and streptavidin-labeled XL665 are used to bring the fluorophores into proximity as a measure of the amount of phosphorylation. Ab, antibody, EuK, europium cryptate.*

Both EuK and proximal (bound) XL665 are measured simultaneously in the Discovery® HTRF Microplate Analyzer (Packard) in black, low fluorescent 96- or 384-well microplates. While the individual fluorescence counts from XL665 (665 nm) and EuK (620 nm) are important, the primary measurement is the ratio (665/620). The Ratio is multiplied by an arbitrary number, typically 10,000. This is simply for convenience of manipulation and has no effect on statistical validity or accuracy. The counts reported by the Discovery are the summation of 20 readings over a one-second time period. HTRF data can be presented in several ways, see Box 2.

Box 2. HTRF data handling

$$R\ (\text{Ratio}) = \text{Counts}_{665\ nm}/\text{Counts}_{620\ nm} \quad (1)$$

$$\Delta R = R_{sample} - R_{blank} \quad (2)$$

$$\Delta F = (R_{sample} - R_{blank})/R_{blank} = \Delta R/R_{blank} \quad (3)$$

$$\Delta F\% = (\Delta R/R_{blank}) \times 100 \quad (4)$$

Typical HTRF assay strategy

The labeling strategy for a typical tyrosine kinase assay using HTRF is shown in Figure 2. The kinase substrate used in this example is a synthetic polymer of glutamic acid, alanine and tyrosine (poly-Glu-Ala-Tyr) that has been biotinylated. Other substrates have been used successfully, including biotin–poly-Glu-Tyr, specific biotin–peptides and XL665–peptides (directly labeled with XL665 without the use of biotin). The substrate is mixed with the tyrosine kinase in the presence of kinase assay components such as ATP, Mg^{2+} and Mn^{2+}. After incubation, a commercially available antiphosphotyrosine antibody (for example, PY20, Transduction Laboratories, Lexington, KY, USA) labeled with europium cryptate, and streptavidin labeled with XL665 are added. A phosphorylated substrate would place the EuK and XL665 in proximity and generate the long-lived signal at 665 nm. The same substrate and antibody that are used for colorimetric or radioisotopic methods can also be used for HTRF.

Optimizing reagent concentrations

The kinase assays differ in the source of enzyme and substrate, so the optimized assay conditions, counts,

fluorescence ratios and signal-to-noise (S/N) ratios will vary. Each assay reported in this review has been characterized by studying several variables including concentration of the EuK-labeled antibody, XL665-labeled peptide, tyrosine kinase concentration, kinetics, ATP dependence and response to inhibitors. Although there are some differences in the assay conditions, the general assay procedures and the signal generation method are very similar. Several variations on the kinase protocol as well as representative results that illustrate the properties of the kinase assay are presented below.

Assay procedures

Receptor tyrosine kinase assay using HTRF

An example of a receptor kinase assay developed at Rhône-Poulenc Rorer is the phosphorylation of substrate using the epidermal growth factor receptor (EGFR) as a source of tyrosine kinase. The EGFR was isolated from A431 cells by homogenization and centrifugation. No unusual purification steps were required. Initially 10 μl of EGFR was preactivated by incubation with EGF for 10 min at room temperature in 60 mM Tris-Mes (pH 7.4) buffer immediately before use. Then 10 μl of biotinylated poly-Glu-Ala-Tyr (final concentration 1 μM) and 30 μl Tris-Mes buffer containing 6 mM Mn^{2+} and 30 mM Mg^{2+} was added. Finally, after an incubation of 20 min at room temperature, 250 μl of phosphate buffer (pH 7.0) containing XL665-labeled streptavidin (2 μg), EuK labeled antiphosphotyrosine antibody (EuK–Ab; 25 ng) and 400 mM KF was added. Fluorescence was measured with the Discovery for one second per well after a 30 min incubation. The KF is added to HTRF assays to increase the fluorescence signal by reducing Eu^{3+} non-radiative deactivation.

Comparing HTRF with SPA and miniaturization

Experiments were designed at Merck to compare HTRF with an SPA kinase assay previously developed[11]. The phosphorylation portion of the kinase assay was similar for HTRF or SPA. The assay buffer was 50 mM Hepes (pH 7.0) with 10 mM $MgCl_2$, 0.1% BSA (bovine serum albumin) and 1 mM dithiothreitol. Biotinylated substrate at 2 μM was used in both assays. The reagent concentrations that differed in this reaction were ATP (2 μM for SPA, 10 μM for HTRF) and kinase (1–4 nM for SPA, 0.02–0.05 nM for HTRF).

After incubation, the HTRF reaction was quenched and the signal developed by the addition of streptavidin–XL665 (420 nM) and EuK–Ab (0.4 nM) in 50 mM Hepes (pH 7.3) with 30 mM EDTA, 0.1% Triton X-100, 0.1% BSA and 400 mM KF. The signal was measured with the Discovery.

For SPA, the reaction was quenched with the addition of 1 mg streptavidin–SPA beads in 50 mM Hepes (pH 7.3) with 30 mM EDTA, 0.1% Triton X-100 and 0.1% BSA. After centrifugation the samples were analyzed on the TopCount® HTS (Packard).

Assay miniaturization in 384-well microplates was tested using both HTRF and SPA. Reaction conditions were the same as noted above except that lower volumes of reagents were used. The samples were measured on the Discovery (HTRF) or TopCount HTS (SPA).

Comparing HTRF with a heterogeneous fluorescence format

HTRF was compared with a heterogeneous (in-plate binding) time-resolved fluorescence (trf) format for a ZAP-70 kinase assay at Glaxo Wellcome. An N-terminal biotinylated peptide substrate was synthesized in-house and the tyrosine kinase was expressed in Sf-9 insect cells. 100,000*g* supernatants were prepared from cell lysates and stored at −80°C. Prior to use, the enzyme preparation was diluted to ~0.1–0.3 mg ml^{-1} in 100 mM Hepes (pH 7.4) and 20 μl aliquots were added to 10 μl of 10% dimethylsulfoxide, plus or minus test compound, and incubated for 15 min at room temperature. The reaction was started by the addition of 20 μl of solution containing $MnCl_2$, biotinylated peptide substrate and ATP. The reaction was incubated at room temperature for 30 min and terminated by the addition of 50 μl of 40 mM EDTA (pH 7.4). The phosphorylation reaction was the same for either HTRF or the heterogeneous trf method. The procedures differed from this point.

HTRF assay procedure. Added directly to the enzyme reaction mixture were EuK–Ab (PY-20, 0.25 ng) and XL665-streptavidin (500 ng) in 100 μl of 20 mM phosphate buffer (pH 7.4) containing 800 mM KF and 0.2% BSA. After a minimum incubation of 30 min the plates were read on the Discovery. The values remained stable for up to seven days.

Heterogeneous trf procedure. A portion (5–20 μl) of the enzyme reaction mixture previously described was transferred to a streptavidin-coated 96-well plate (Boehringer Mannheim) containing assay buffer (Wallac Oy) to give a final volume of 100 μl. The biotinylated peptide was

allowed to bind for 20 min. Blank wells were prepared by the addition of EDTA prior to the addition of the final reagent mixture and the mean value was subtracted from the respective experimental wells. The plate was washed three times under running cold water and gently tapped dry. Europium labeled antiphosphotyrosine antibody (PT-66, 5–8 Eu per IgG, 120 ng protein well^{-1}) was added in 100 μl assay buffer and incubated at room temperature for 15 min. The plate was washed five times under running cold water and tapped dry. Enhancement solution (200 μl, Wallac) was added to each well and the plate was left to stand for a minimum of 20 min and read in the time-resolved fluorescence mode in a Wallac 1420 plate reader. The values remained stable for up to seven days.

Subsets of the Glaxo Wellcome Compound Register biased towards known kinase inhibitors were selected for evaluation against ZAP-70 in both HTRF and trf formats. Each compound was screened in duplicate at 20 μM with 40 compounds, eight controls and eight blanks in each plate.

Optimizing reagent concentrations

Fluorescent-labeled assay components

The optimization of the fluorescence-labeled reagents, XL665–peptide and EuK–Ab (Figure 3a and 3b, respectively), was determined at Boehringer Ingelheim[12]. A final concentration of 0.15 μM was selected for XL665–peptide. This concentration produced a good signal and S/N ratio and still retains sensitivity to inhibition. The format for this particular assay used a peptide substrate that was directly coupled to XL665 instead of using a biotinylated substrate. The EuK–Ab gave an increasing signal up to the highest concentration tested. The final concentration that was selected (0.8 nM) provided a good S/N ratio, minimized reagent use and had sufficient counts at 620 nm to serve as an internal reference signal.

ATP dependence

ATP is the source of phosphate for substrate phosphorylation and it is an important parameter in the assay. Figure 4 shows the dependence of ATP on the EGF receptor associated kinase as studied at Rhône-Poulenc Rorer. A K_m of 3.64 μM was calculated. These results are in accordance with results obtained by J. Boiziau (Rhône-Poulenc Rorer) using an ELISA format (pers. commun.). HTRF kinase assays can be run at ATP concentrations up to several hundred μM, but are typically run near the K_m concentration.

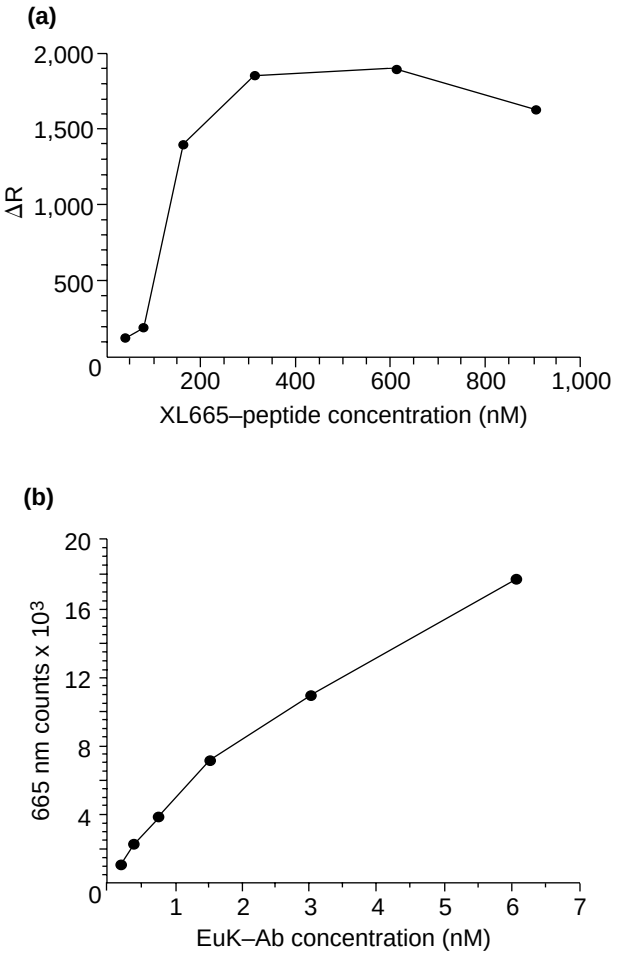

Figure 3. *The effect of fluorescent substrate concentrations on phosphorylation. The concentrations of (a) XL665–peptide substrate and (b) europium cryptate–antiphosphotyrosine antibody (EuK–Ab) were optimized. As with any assay method, a balance was found between counts, signal to noise and reagent use. Working concentrations of 150 nM XL665–peptide and 0.8 nM EuK–Ab were selected (Boehringer Ingelheim). ΔR is calculated as described in equation 2.*

Enzyme optimization

A detailed kinetics of lck tyrosine kinase was studied at Merck[11] and is shown in Figure 5. Concentrations of kinase as low as 5 pM showed a linear response over one hour and could be measured over the blank (time zero), although at a low S/N ratio. A S/N ratio of >6 could be obtained in a 15 min incubation using 0.2 nM lck. These data show the ability to select an enzyme concentration

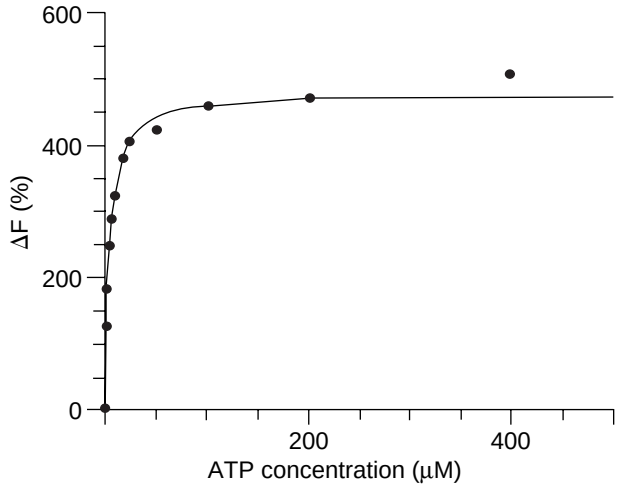

Figure 4. *ATP dependence of the epidermal growth factor (EGF) receptor kinase. The EGF receptor-associated kinase was isolated from A321 cells. Using a biotin–poly-Glu-Ala-Tyr substrate, a* K_m *for ATP of 3.64 μM was calculated (Rhône-Poulenc Rorer). ΔF is calculated as described in equation 4.*

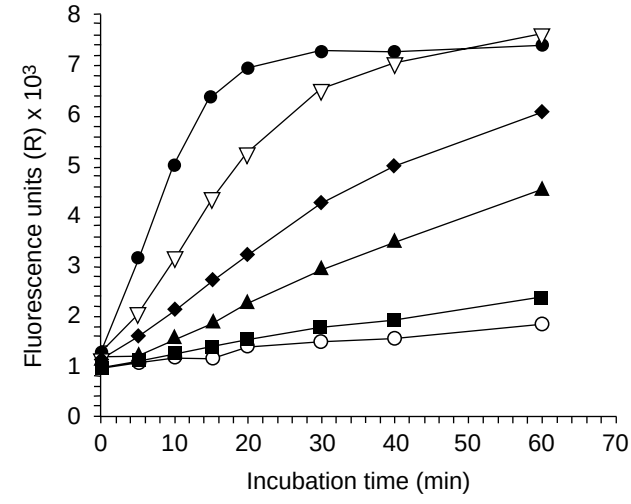

Figure 5. *Kinetics of substrate phosphorylation. The assays as developed at Merck were carried out in 96-well plates in 200 μl volumes. Final reagent concentrations included 0.4 nM EuK–Ab, 420 nM streptavidin–XL665 and 500 nM substrate with lck tyrosine kinase concentrations as follows: white circles, 5 pM; black squares, 10 pM; black triangles, 25 pM; black diamonds, 50 pM; white triangles, 100 pM; black circles, 200 pM.*

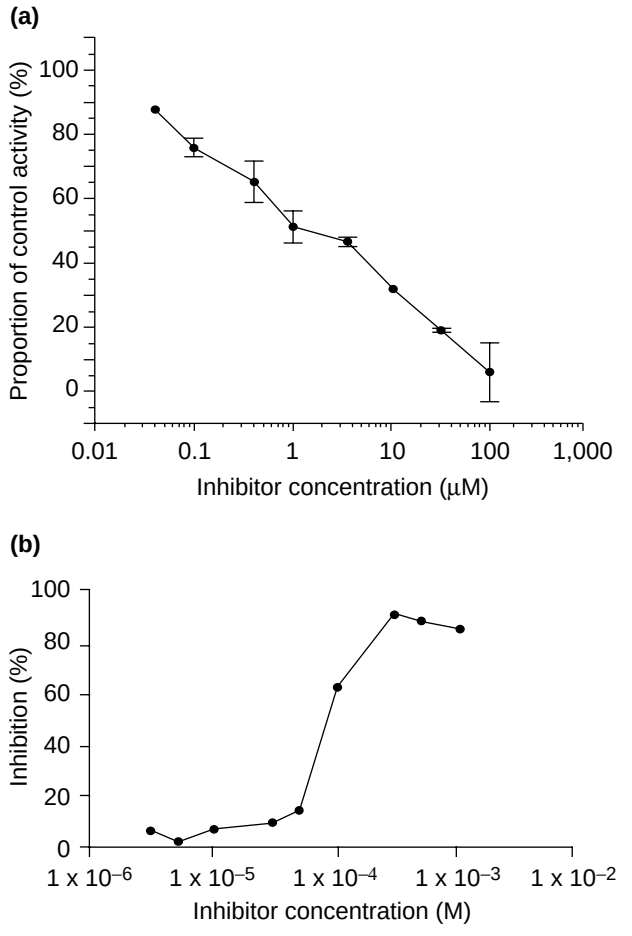

Figure 6. *Inhibition of tyrosine kinase activity. (a) Boehringer Ingelheim experiments showing the inhibition of a tyrosine kinase by a moderate inhibitor with an* IC_{50} *of 1 μM. (b) Zeneca Pharmaceuticals identification of an extremely weak inhibitor with an* IC_{50} *of ~100 μM. Error bars show SEM.*

that balances the S/N ratio with the cost of the reagent and the incubation time. If reagent conservation is important, a minimum concentration can be used that still produces a sufficient signal above background to meet the laboratory criteria. If the enzyme is available in large quantities, a greater concentration can be added to increase the signal and decrease the incubation time. This also has an impact on miniaturization, as discussed in a later section.

Identification of inhibitors

The purpose of any screening assay is to identify compounds of potential therapeutic value. Experiments at Boehringer Ingelheim (Figure 6a) and Zeneca (Figure 6b)

illustrate the ability of HTRF to detect even very weak inhibitors. The IC_{50} values are approximately 1 μM and 100 μM, respectively. Control plates with replicates of maximum and minimum phosphorylation gave CV values of 5%, thus allowing the identification of weak inhibitors.

The ability to identify low-affinity inhibitors is becoming more important in HTS as the targets become more challenging. Therapeutic targets with low affinities are becoming more common and to be successful as high-throughput screens they will require sensitive assay methods. HTRF can measure lower affinity binding than methods that require separation steps or attachment to solid supports.

Assay miniaturization and a comparison of HTRF with SPA

Assay miniaturization is becoming necessary to meet the goals of HTS. The feasibility of performing radioisotopic and luminescence assays in 384-well microplates has been demonstrated[13], and fluorescence quench assays in 384, 864 and higher well densities have also been reported[14].

To test the miniaturization of HTRF for a screening assay, a lck tyrosine kinase assay optimized by Merck was used. Figure 7 shows the results of experiments using 96-well plates (200 μl) and 384-well plates (65 μl and 32.5 μl). The HTRF S/N ratio shows a similar response at each volume for lck kinase concentrations from 10 pM to 100 pM. The S/N ratio using 100 pM lck ranges from ~8 (384-well plate, 32.5 μl) to ~12 (96-well plate, 200 μl)[11].

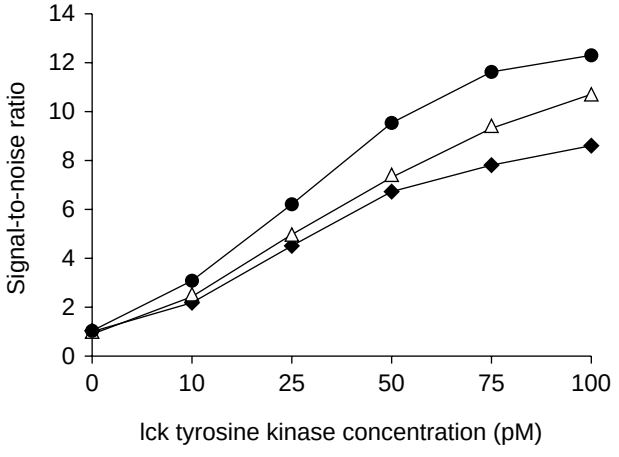

Figure 7. *A comparison of signal-to-noise ratios for a lck tyrosine kinase in 96- and 384-well formats. Samples were counted in 96-well (200 μl, circles) or 384-well (65 μl, triangles and 32.5 μl, diamonds) HTRF plates using six concentrations of lck kinase (Merck).*

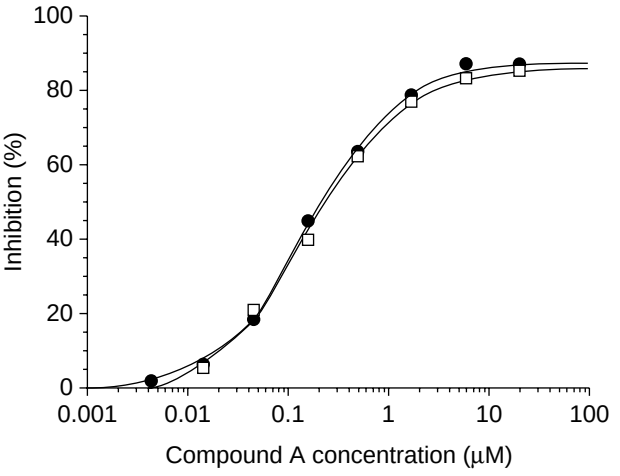

Figure 8. *Comparison of inhibitors using HTRF and SPA. Inhibition (%) was calculated for a known inhibitor of lck kinase using HTRF (circles) and SPA (squares) in 96-well microplates as described using the Merck protocol. The SPA samples were centrifuged to pellet the beads and avoid variations resulting from the beads settling with time. The IC_{50} for this strong competitor (0.152 μM, HTRF and 0.172 μM, SPA) was similar using either method (Merck).*

As SPA is a common radioisotopic technology used in drug discovery, it is appropriate to compare the results of this method with HTRF. Figure 8 shows the results of a comparison between HTRF and SPA in the 96-well format for a known inhibitor of lck. The results were essentially identical with IC_{50} values of 0.152 μM (HTRF) and 0.172 μM (SPA). The SPA samples were centrifuged to pellet the beads, which avoids the problem of counts changing as the beads settle with time.

The HTRF and SPA comparisons were expanded to include miniaturization to the 384-well format. Figure 9 shows the results of 96-well and 384-well assays using HTRF and SPA. Both assays miniaturized easily with little change in the S/N from the 96-well (200 μl) to the 384-well format (32.5 μl for HTRF, 50 μl for SPA). The assay conditions were optimized for each method to balance the S/N and reagent concentrations. The amount of substrate was the same (2 μM), but the amount of enzyme used in the SPA (2 nM) was 40 times higher than that required for HTRF (50 pM). The amount of ATP that could be used in the SPA was limited because ATP also acts as the source of

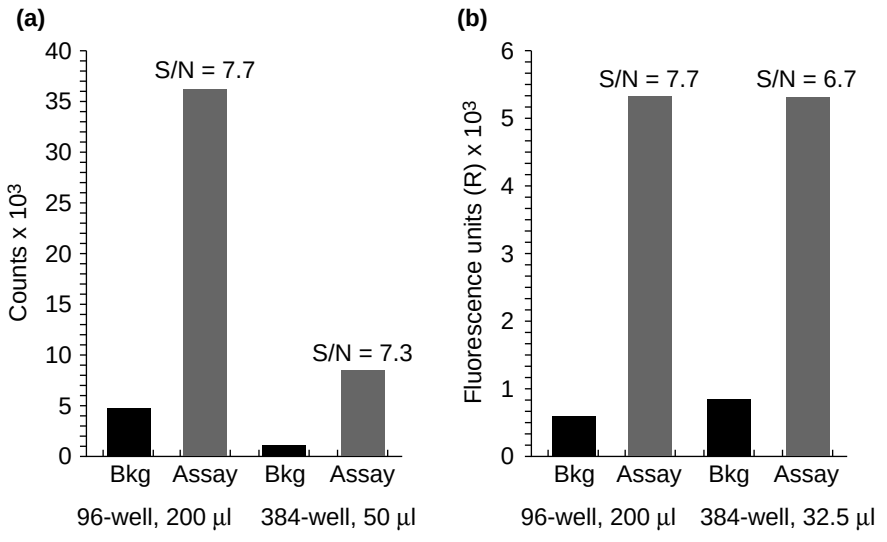

Figure 9. *Miniaturization of HTRF and SPA tyrosine kinase assays. Kinase assays were tested in the 96-well and 384-well formats using (a) HTRF or (b) SPA. The assay components for HTRF were 10 µM ATP, 2 µM substrate and 50 pM lck tyrosine kinase in 200 µl (96-well) or 32.5 µl (384-well). For SPA the components were 2 µM ATP, 2 µM substrate and 2 nM lck in either 200 µl (96-well) or 50 µl (384-well) (Merck). Bkg, background; S/N, signal-to-noise ratio.*

^{33}P. That is to say, the [^{33}P]ATP concentration must be limited for cost and safety reasons and the unlabeled ATP concentration has to be limited to achieve maximum incorporation of the ^{33}P. The HTRF assay could use a higher ATP concentration (10 µM) than SPA (2 µM) to optimize phosphorylation.

HTRF and fluorescence methods in general have a significant advantage over radioisotopic assays in miniaturization. The radioactive signal decreases in proportion to the volume, assuming the same reagent concentrations. Hence, with lower counts, the sample must be counted longer to achieve the same statistical accuracy. As throughput is already a concern with radioisotopic assays, miniaturization will only increase this problem. The signal from fluorescence, however, is dependent upon the number of molecules excited. For a fixed laser beam diameter, as used for excitation in the Discovery, the number of molecules excited is a function of pathlength. Although there are geometry and optical considerations affecting light collection, the fluorescent signal decreases proportionally less than the sample volume. The large number of photons produced from the excitation of fluorescent sample and the ability to excite the same sample multiple times (20 times per second in the Discovery) makes the miniaturization of fluorescence methods more practical.

Comparison of HTRF with in-plate time-resolved fluorescence

Researchers at Glaxo Wellcome with experience of an in-plate time-resolved fluorescence (trf) assay (Delfia®, Wallac Oy), compared this method with HTRF. The effect of enzyme concentration on signal intensity was investigated and the results are shown in Figure 10. Because of the different units used in the two methods (a ratio in HTRF and arbitrary fluorescence units in trf), the results are plotted as a percentage of the maximum signal. For example, the highest ratio for HTRF was at 2.5 µg well^{-1} of the ZAP-70 kinase. This was the 100% value for the HTRF data. The optimum HTRF signals were reached at a lower enzyme concentration (2.5 µg well^{-1}) than the corresponding trf assay (5 µg well^{-1}). A concentration of 5 µg well^{-1} was used in subsequent assays, although this was not optimal for HTRF.

The activity of ZAP-70 was titrated against staurosporine, a known kinase inhibitor, and the inhibition curves are shown in Figure 11. Potent inhibition was achieved and the IC_{50} values were essentially the same for both methods (22 nM for HTRF and 31 nM for trf). The difference in the slope values (1.69 for HTRF and 0.87 for trf) may have resulted from the use of two different antiphosphotyrosine antibodies.

The lack of plate transfers and washing steps made the HTRF assay much more convenient to prepare. The HTRF assay took ~8 h to prepare, incubate and count 3,360 samples, while the trf assay took ~14 h for only 1,600 samples. The sample set contained several colored compounds. The trf showed no problems with color quenching, often referred to as the inner filter effect, because of the transfer and washing steps. HTRF also showed no color quenching, even though it is a homogeneous assay, because of the correction resulting from the use of the 665/620 wavelength

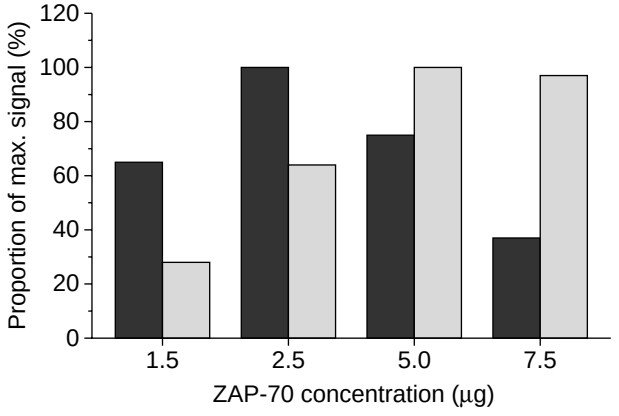

Figure 10. *Effect of ZAP-70 enzyme concentration on HTRF and heterogeneous time-resolved fluorescence (trf) signals. A comparison was made of the ZAP-70 kinase concentration required for HTRF (black bars) or heterogeneous trf (grey bars) assays as described under the Glaxo Wellcome protocol. Results are plotted as a percentage of the maximum signal. HTRF samples were measured on Discovery and trf samples on the Wallac 1420.*

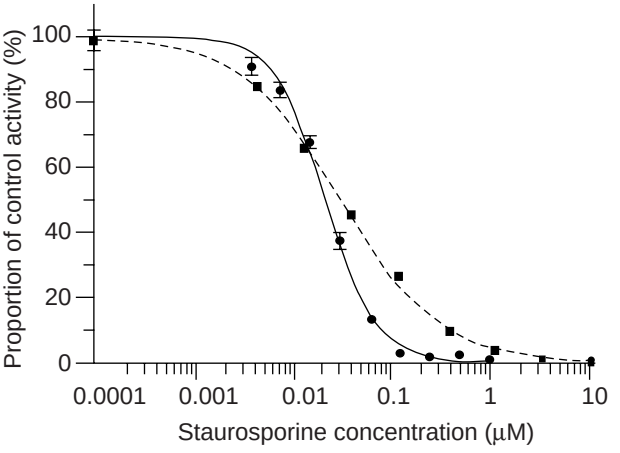

Figure 11. *Response of HTRF and heterogeneous time-resolved fluorescence (trf) methods to ZAP-70 inhibition by staurosporine. This known inhibitor of tyrosine kinase was found to give similar IC_{50} values of 22 nM (HTRF, circles) and 31 nM (trf, squares) under the Glaxo Wellcome assay conditions. The difference in slopes may be attributed to the different assay conditions and the use of different antiphosphotyrosine antibodies.*

ratio. The hit rate was 1.5% for HTRF and 2.5% for trf. The hit rate can be adjusted higher or lower by changing the concentrations of substrate and ATP.

In terms of the S/N ratio (defined as control values/blank values), the trf had a higher value (15:1) than HTRF (6:1). In preliminary HTRF experiments that were designed to give a low S/N by using a suboptimal enzyme concentration, standard inhibitors produced good titration curves at a S/N ratio as low as 2:1. So although a S/N ratio of 15:1 looks superior to 6:1, in practice it makes no difference, and the greater ease of performing HTRF outweighs any perceived advantage in S/N.

Conclusion

Comparisons of assay technologies include labor, throughput, robustness, hit rates and artifacts that result in false positives or false negatives. Some of these measures can be more easily quantitated than others. For example, labor and throughput are relatively easy to measure. HTRF, being a homogeneous method with fast measurement times, has advantages over techniques that require separation or the use of radioactivity. Other criteria, are more subjective and difficult to measure. For example, hit rates have more variability because they can be adjusted by reagent concentrations and inhibitor cut-off values. Using this measure, HTRF is comparable with other methods. Artifacts that result in false positives or false negatives are probably the most difficult and often the most subjective measure. It is common to compare the hits from a new assay technology with those found by the established method. The assumption is that the hits found in the first method are correct, although this is not necessarily accurate. HTRF has verified the known inhibitors found by other methods. As far as artifacts are concerned, every method comes with a set of advantages, disadvantages and conditions that affect performance. Radioisotopic assays have several interferences that decrease efficiency, especially color quenching in non-separation methods. In-plate assays can be affected by conditions that strip the plate coatings and by variability in the wash procedures. HTRF is largely self-correcting for color and does not require wash steps, but compounds that interfere with the energy transfer process will affect the results. All these methods have conditions that result in some level of false positives and false negatives. In a wide variety of assays and sources of test compounds (natural products and chemical libraries), HTRF has shown little susceptibility to artifacts.

One point that is agreed upon is that HTS is a challenging area of drug discovery. Scientists in screening are being asked to run more targets per year and more compounds per target with only a small increase in staff and total budget. Along with the usual requirements of robustness and simplicity, this will require a sensitive assay technology that offers high throughput with minimum labor. HTRF has these characteristics and they have been proven with a variety of targets. The results reported in this review are typical for assays being adapted to HTRF. In addition to tyrosine kinase assays[7,11,12], HTRF has been adapted to a wide range of biomolecular interactions including immunoassays[8], protein–protein binding[7], viral protease[15,16], receptor binding[7] and nucleic acid hybridization[17]. Screening will probably require a mix of assay technologies including radiometric, colorimetric, luminescent and several fluorescent methods. For HTRF, low reagent concentrations, simplified assay protocols, robustness under screening conditions, the ability to detect weak inhibitors, miniaturization, compensation for color absorption from natural products, sensitivity, safety and speed are often cited as advantages. With throughputs in the range of 50,000 wells per day with one Discovery microplate fluorometer, HTRF is becoming one of the assay methods that will help HTS meet the increasing demands of the future.

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In short…

Hewlett-Packard Co. (HP) and **Caliper Technologies Corp**. have signed an agreement for the joint development of miniaturized analytical instrumentation and information systems based on Caliper's LabChip™. Over the next year they expect to invest $20 million for the development of the first-generation instruments, and intend to invest a further $80 million, over the next four years, towards additional development and commercialization of the technology.

The new system will integrate a plethora of chemical manipulations on a single chip; digital data derived from the chips will be stored in databases that can be accessed via networks and shared by research scientists. The new system is expected to enhance the speed and reduce the cost of chemical analysis by several orders of magnitude – the speed of analysis will be measured in chemical interactions per second and the costs for each analysis are expected to drop from dollars to mere cents. The worldwide market for these microfluidics-based analytical systems is expected to be $1 billion early in the next century.

Rick Kniss, HP Vice President and General Manager for their Chemical Analysis Group, says 'we will develop instrumentation and chips that will help our customers, especially R&D scientists in pharmaceutical labs, to slash their R&D time. We expect a tenfold increase in the quality and amount of information generated as a result of this technology.' Calvin Chow, Caliper's Chief Operating Officer, says that 'no other company matches HP's leadership in engineering and information technology or its deep experience in the analytical-instrumentation area. HP is the perfect partner for Caliper's breakthrough technology.'